A Novel Synthetic Vitamin A-like Compound (A Polyprenoic Acid Derivative, E-5166) Inhibits the Release of Arachidonic Acid Stimulated by Epidermal Growth Factor

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Little is known about the mechanisms of anti-inflammatory activity of retinoids. A new synthetic vitamin A-like compound (polyprenoic acid derivative, E-5166) has a strong in vitro binding affinity to intracellular binding proteins for acidic retinoids. In order to elucidate the anti-inflammatory activity of E-5166, we studied the effect of E-5166 on the epidermal growth factor (EGF)-stimulated arachidonic acid (AA) release of pig epidermis. E-5166 significantly inhibited the EGF-stimulated AA release and this inhibitory effect of E-5166 required a longer incubation than hydrocortisone did. Furthermore, E-5166 inhibited the EGF-stimulated phosphatidylinositol (PI) turnover of pig epidermis. These results indicate that E-5166 inhibited the EGF-stimulated AA release through the inhibition of the EGF-stimulated PI turnover. *J Invest Dermatol 88:630–633, 1987*

Vitamin A and its synthetic analogs (retinoids) have been shown to control cellular differentiation and proliferation in many tissues [1]. Besides the inhibition of cell proliferation, the effects of retinoids in cystic acne and psoriasis have been attributed to their anti-inflammatory action [2,3]. It has been postulated that the anti-inflammatory activity might be due to inhibition of the production of arachidonic acid (AA)-derived mediators of inflammation, but there are few reports dealing with the ability of retinoids to inhibit AA metabolism [4].

A new synthetic vitamin A-like compound (3,7,11,15-tetramethyl-2,4,6,10,14-hexadecapentaenoic acid, E-5166), which was originally described by Muto et al [5,6], had a strong in vitro binding affinity to intracellular binding proteins for acidic retinoids. In order to elucidate the anti-inflammatory action of E-5166, we studied the effect of E-5166 on the AA metabolism, particularly the phospholipid-breakdown system of pig epidermis. As we have already reported that epidermal growth factor (EGF) and other phospholipase A_2 (PLase A_2) stimulators increased the AA release from membrane phospholipids of pig epidermis [7], we studied the effect of E-5166 on the EGF-stimulated AA release of pig epidermis.

In this paper, we report that E-5166 significantly inhibited the EGF-stimulated AA release and this inhibitory effect of E-5166 required a longer incubation than HC did. Furthermore, we discuss different mechanisms of action of E-5166 and HC on the inhibition of the AA release in pig epidermis.

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Abbreviations:
AA: arachidonic acid
A23187: ionophore A23187
BSA: bovine serum albumin
DG: diglycerol
EGF: epidermal growth factor
E-5156: 3,7,11,15-tetramethyl-2,4,6,10,14-hexadecapentaenoic acid
HC: hydrocortisone
KRB: Krebs-Ringer buffer
PA: phosphatidic acid
PC: phosphatidylcholine
PE: phosphatidylethanolamine
PI: phosphatidylinositol
PLase A_2: phospholipase A_2
PS: phosphatidylserine
TPA: 12-*O*-tetradecanoylphorbol-13-acetate

MATERIALS AND METHODS

Skin slices were taken from the backs of domestic pigs, weighing 6–8 kg, by a Castroviejo keratome set at 0.2 mm depth. As skin slices thus obtained contain mainly epidermis (80–90%), they are called epidermal slices [8]. During procedure, pigs were anesthetized with Nembutal (20–30 mg/kg) i.p. Epidermal slices kept in RPMI 1640 medium at 4°C were then cut into 5 × 10 mm and used within 30 min.

Epidermal slices were pretreated with or without E-5166 or hydrocortisone (HC) for indicated periods, and then prelabeled with [1-^{14}C]arachidonic acid ([^{14}C]AA) (0.1 μCi/ml) for 4 h in RPMI 1640 medium with 2% bovine serum albumin (BSA) at 37°C in a high-humidity incubator in a mixture of 5% CO_2 in air.

After prelabeling, epidermal slices were washed twice in Ca^{++}-free RPMI 1640 medium with 1 mM ethyleneglycoltetraacetic acid, and then incubated with or without EGF or other test drugs in Ca^{++}-free RPMI 1640 with 1.2 mM Ca^{++} and 2% BSA at 37°C for 20 min. The incubation was terminated by removing samples from the medium immediately. The radioactivity of [^{14}C]AA released in the medium was determined as previously

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described [7]. In this experiment system, we did not detect any significant difference of ^{14}C-labeled AA metabolites released in the medium between control and E-5166 treatment. The AA content in the medium was more than 85% of the total activity.

For the study of phospholipid breakdown, epidermal slices pretreated with or without E-5166 for 22 h were prelabeled with [^{3}H]glycerol (2 μCi/ml) for 1 h and then incubated with or without EGF for indicated periods. The incubation medium was the modified Krebs-Ringer buffer (KRB) with HEPES (20 mM, pH 7.4) in the presence of 1.2 mM Ca^{++}. The samples were rinsed in a chilled KRB and immediately frozen between 2 dry-ice plates to terminate the reaction.

The samples were then homogenized with a glass homogenizer in 3 ml of chloroform:methanol (2:1) or methanol at 4°C. After homogenization, 1 ml of 2 M KCl solution was added and the upper phase was removed. The extract was evaporated to a small volume with nitrogen gas. Lipids and phospholipids labeled with [^{3}H]glycerol were dissolved in chloroform and analyzed by thin-layer chromatography (precoated Silica Gel G Plate, Wako Chemicals, Osaka, Japan) as described by Snyder [9]. The solvent system contained chloroform:methanol:acetic acid:H_2O (50:25:8:4) for phospholipids and ligraine:ethanol:acetic acid (50:50:1) for lipids. Each spot of lipids and phospholipids was scraped and its radioactivity was determined by a scintillation counter.

For the study of [^{3}H]inositol incorporation into phosphatidylinositol (PI), a long incubation system was employed. Epidermal slices pretreated with or without E-5166 for 22 h were washed twice and then incubated with or without EGF in the presence of [^{3}H]inositol (1 μCi/ml) for 1 h. The incubation medium was KRB with HEPES (20 mM, pH 7.4) in the presence of Ca^{++} (1.2 mM). The samples were homogenized and phospholipid fraction was extracted. The radioactivity of an aliquot of phospholipid fraction labeled with [^{3}H]inositol was determined by a scintillation counter as described previously [10].

Protein content was measured by the method of Lowry et al [11].

The following chemicals and drugs were obtained from the indicated sources: [^{3}H]glycerol, [^{3}H]inositol, and [^{14}C]AA (Amersham Japan, Tokyo, Japan); EGF (Collaborative Research Inc., Waltham, Massachusetts); Ionophore A23187 (A23187) (Calbiochem-Behringer Co., San Diego, California); HC, 12-O-tetradecanoylphofbol-13-acetate (TPA), phosphatidylethanolamine (PE), phosphatidylcholine (PC), phosphatidylserine (PS), and BSA (essential fatty acid–free) (Sigma Chemical Co., St. Louis, Missouri); and RPMI 1640 and Ca^{++}-free RPMI 1640 media (GIBCO, Grand Island, New York). E-5166 was a generous gift of Eisai Co., Ltd., Tokyo, Japan. All other chemicals and drugs were analytic reagent grade.

RESULTS

As shown in Table I, E-5166 at 100 μM had no detectable effect on the AA release stimulated by either A23187, EGF, or TPA,

Table I. Effect of E-5166 on the AA Release Stimulated by A23187, EGF, and TPA

	AA Release (dpm/mg protein/20 min)	
	Control	E-5166 (100 μM)
None	175 ± 12.9	160 ± 2.7
A23187 (10 μM)	500 ± 34.7	455 ± 50.1
EGF (0.1 μg/ml)	243 ± 1.1	244 ± 2.6
TPA (1 μM)	277 ± 5.0	254 ± 3.6
Total incorporation	4,250 ± 174.0	4,291 ± 37.3

Epidermal slices prelabeled with [^{14}C]AA (0.1 μCi/ml) were incubated with or without E-5166 (100 μM) in the presence or absence of A23187 (10 μM), EGF (0.1 μg/ml), or TPA (1 μM) for 20 min. The amount of [^{14}C]AA released in the medium was determined and expressed as dpm/mg protein ± SEM. Number of samples = 3. Total incorporation of [^{14}C]AA into epidermal slices was determined and expressed as dpm/mg protein ± SEM.

Table II. Effect of E-5166 on the AA Release Stimulated by A23187, EGF, and TPA

	AA Release (dpm/mg protein/20 min)	
	Control	E-5166 (100 μM)
None	255 ± 25.0	199 ± 18.6
A23187 (10 μM)	682 ± 71.8	460 ± 74.8
EGF (0.1 μg/ml)	455 ± 30.4	236 ± 31.7[a]
TPA (1μM)	486 ± 49.4	303 ± 31.9[b]
Total incorporation	5,284 ± 425	5,077 ± 343

Epidermal slices prelabeled with or without E-5166 (100 μM) for 22 hr were incubated with [^{14}C]AA (0.1 μCi/ml), and then incubated with or without A23187 (10 μM), EGF (0.1 μg/ml), and TPA (1 μM) for 20 min. The amount of [^{14}C]AA released in the medium was determined and expressed as dpm/mg protein/20 min ± SEM. Total incorporation of [^{14}C]AA into epidermal slices was determined and expressed as dpm/mg protein ± SEM. Number of samples = 6.

[a] $p < 0.01$ (compared with control).

[b] $p < 0.025$ (compared with control).

when it was simultaneously added to the incubation medium with PLase A_2 stimulators.

When epidermal slices were pretreated with E-5166 at 100 μM for 22 h, it significantly inhibited the AA release stimulated by EGF as shown in Table II. The TPA-stimulated AA release was also inhibited by the pretreatment with E-5166, but the A23187-stimulated one was not. A prelabeling for 4 h with [^{14}C]AA resulted in almost the same amount of ^{14}C incorporation into total phospholipids of the control or E-5166-treated epidermal slices.

As shown in Fig 1, E-5166 required at least 6 h to inhibit the EGF-stimulated AA release. E-5166 at 100 μM induced 60% inhibition of the EGF-stimulated AA release by 22-h pretreatment.

Figure 2 shows a dose-dependent study of E-5166 effect. E-5166 at less than 10 μM had no effect, but 100 μM and 1 mM of E-5166 inhibited the EGF-stimulated AA release.

As shown in Fig 3, the addition of cycloheximide at 1 μg/ml, an inhibitor of protein synthesis, partially eliminated the E-5166-dependent inhibitory effect of the EGF-stimulated AA release.

As shown in Fig 4, HC alone at 1 μM or E-5166 alone at 100 μM significantly inhibited the EGF-stimulated AA release. The presence of HC additively potentiated the inhibitory effect of E-5166 on the EGF-stimulated AA release. Hydrocortisone at 10

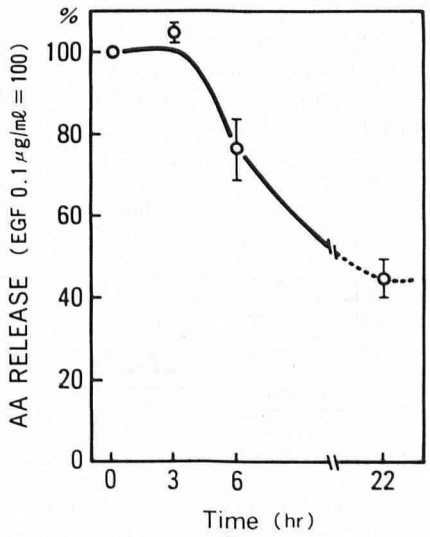

Figure 1. Effect of E-5166 on the EGF-stimulated AA release (time course study). Epidermal slices pretreated with or without E-5166 (100 μM) for indicated periods were prelabeled with [^{14}C]AA (0.1 μCi/ml) for 4 h, and then incubated with EGF at 0.1 μg/ml for 20 min. The amount of [^{14}C]AA released in the medium was determined and expressed as % of 0 time value. Each *bar* represents the standard error of 3 samples.

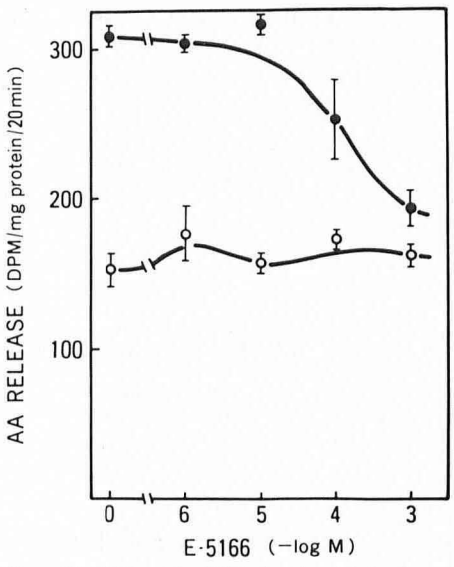

Figure 2. Effect of E-5166 on the EGF-stimulated AA release (dose study). Epidermal slices pretreated with or without E-5166 at various concentrations for 22 h were prelabeled with [^{14}C]AA (0.1 μCi/ml) for 4 h, and then incubated with or without EGF at 0.1 μg/ml for 20 min. The amount of [^{14}C]AA released in the medium was determined and expressed as dpm/mg protein ± SEM. Number of samples = 3; *open circles,* control; *solid circles,* EGF 0.1 μg/ml.

μM also inhibited the EGF-stimulated AA release (55.7 ± 5.5%) and the inhibition was enhanced by E-5166 treatment to 77.3 ± 2.0% (n = 3). In order to further elucidate the action mechanism of E-5166, the effect of phospholipid-breakdown system was studied. In total, in 6 separate experiments the formation of [^{3}H]glycerol-labeled diglycerol (DG) was stimulated by EGF at 5 min to 333 ± 49% of the levels of DG at 0 time. As shown in Fig 5, transient increase of [^{3}H]glycerol-labeled DG formation stimulated by EGF was completely inhibited by pretreatment with E-5166. In this prelabeling system with [^{3}H]glycerol for 1 h, E-5166 slightly inhibited [^{3}H]glycerol labeling of DG, while [^{3}H]glycerol labeling of PA and PI plus PS was stimulated by pretreatment with E-5166 (data not shown).

As shown in Table III, the formation of [^{3}H]inositol-labeled PI was also studied in a continuous labeling system. Epidermal growth factor stimulated the formation of [^{3}H]inositol-labeled PI, while pretreatment with E-5166 inhibited the EGF-stimulated PI formation. In this system, E-5166 enhanced the formation of [^{3}H]inositol labeling of PI.

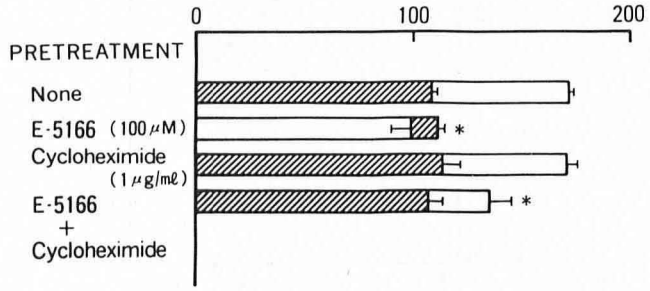

Figure 3. Effect of cycloheximide and/or E-5166 on the EGF-stimulated AA release. Epidermal slices pretreated with or without E-5166 (100 μM) in the presence or absence of cycloheximide (1 μg/ml) for 22 h were prelabeled with [^{14}C]AA (0.1 μCi/ml) for 4 h, and then incubated with EGF at 0.1 μg/ml for 20 min. The amount of [^{14}C]AA released in the medium was determined and expressed as dpm/mg protein/20 min ± SEM. Number of samples = 3. *: $p < 0.01$ (compared with *None*). Control, *shaded areas;* EGF, *open areas.*

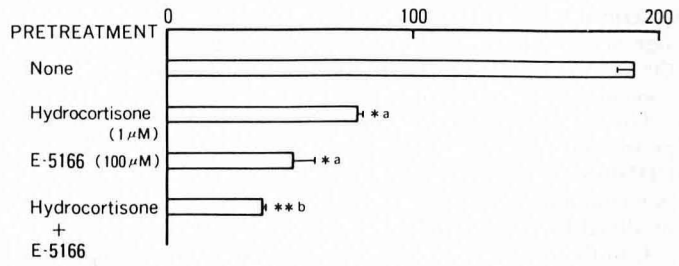

Figure 4. Effect of HC and/or E-5166 on the EGF-stimulated AA release. Epidermal slices pretreated with or without E-5166 (100 μM) in the presence or absence of HC (1 μM) for 22 h were prelabeled with [^{14}C]AA (0.1 μCi/ml), and then incubated with EGF (0.1 μg/ml) for 20 min. The amount of [^{14}C]AA released in the medium was determined and expressed as dpm/mg protein 20 min ± SEM. *a: $p < 0.001$ (compared to *None*). **b: $p < 0.01$ (compared with *Hydrocortisone* or *E-5166*).

DISCUSSION

Like aromatic retinoids, E-5166 is clinically effective, not only for hyperproliferative and dyskeratotic skin diseases but also for inflammatory skin diseases such as psoriasis vulgaris and pustular psoriasis. Although it has been postulated that the anti-inflammatory activity might be due to an inhibition of the production of AA-derived mediators of inflammation, Ruzicka [12] failed to demonstrate the inhibitory activity of aromatic retinoid (Rol0-1670) on either lipoxygenase or cyclooxygenase activities. Further, retinoids have been shown to both increase [13] and decrease [4], or not to affect [14] prostaglandin E_2 synthesis depending upon the type of cells, the retinoid, and its concentration. E-5166 shares a number of biologic and pharmacologic activities with aromatic retinoids, but it was not clear how E-5166 works on inflammatory processes.

We have previously reported that EGF stimulated the release of AA through the activation of PLase A_2 of pig epidermis [7]. The generation of free AA from membrane phospholipids by PLase A_2, which leads to the formation of AA metabolites, is

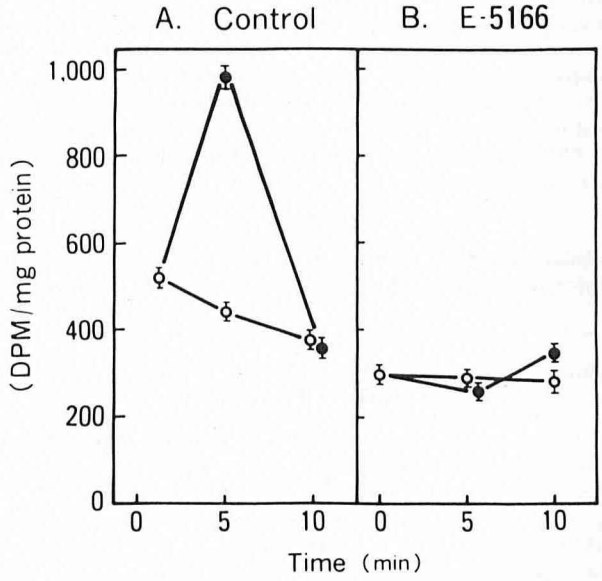

Figure 5. Effect of E-5166 on the EGF-stimulated [^{3}H]glycerol-labeled DG formation. Epidermal slices pretreated with (*B*) or without (*A*) E-5166 (100 μM) for 22 h were prelabeled with [^{3}H]glycerol (2 μCi/ml) for 1 h, and then incubated with or without EGF (0.1 μg/ml) for 10 min. The amount of [^{3}H]glycerol-labeled DG was determined and expressed as dpm/mg protein ± SEM following thin-layer chromatography analysis of lipids extracted from epidermal homogenates. Number of samples = 4; *open circles,* control; *solid circles,* EGF (0.1 μg/ml).

Table III. Effect of E-5166 on the EGF-Stimulated [^{3}H]Inositol Incorporation into PI

	[^{3}H]Inositol Incorporation (dpm/mg protein)	
	Control	E-5166 (100 μM)
None	1,234 ± 199	1,970 ± 303[a]
EGF (0.1 μg/ml)	1,761 ± 134[a]	1,965 ± 252[a]

Epidermal slices prelabeled with or without E-5166 (100 μM) for 22 h were incubated with or without EGF (0.1 μg/ml) in the presence of [^{3}H]inositol (1 μCi/ml) for 1 h. The amount of [^{3}H]inositol incorporated into PI was determined and expressed as dpm/mg protein ± SEM. Number of samples = 6.
[a] $p < 0.005$ (compared with control).

thought to be a rate-limiting process. In order to elucidate the mechanism of action of E-5166 on the inflammatory processes, we studied the effect of E-5166 on the EGF-stimulated AA release of pig epidermis.

E-5166 significantly inhibited the EGF-stimulated AA release. It required at least 6-h incubation and this inhibitory effect of E-5166 was partially eliminated by the addition of cycloheximide, an inhibitor of protein synthesis. Furthermore, the presence of HC additively potentiated the inhibitory effect of E-5166 on the EGF-stimulated AA release.

It has been reported that one of the important actions of corticosteroids on the inflammatory process is to inhibit PLase A_2 activity by synthetizing an inhibitor protein [15,16].

As the activation of PLase A_2 by EGF was preceded by the degradation of PI to DG [10], we were interested in the effect of E-5166 on the EGF-stimulated PI turnover. The EGF induced a transient and significant increase of [^{3}H]glycerol-labeled DG formation, while E-5166 completely inhibited the EGF-stimulated DG formation. The EGF-stimulated PI formation was also inhibited by E-5166.

E-5166 failed to inhibit AA release stimulated by A23187, which activates PLase A_2 activity without the transient formation of PI and PA [17,18]. The release of AA by TPA, which activates protein kinase C directly, was also inhibited by E-5166, but the mechanism by which TPA stimulates AA release is still unknown.

These results indicate that E-5166 inhibited the EGF-stimulated AA release through the inhibition of PI turnover, which is different from the effect of HC, although the precise mechanism of action by which E-5166 inhibits PI turnover was not clearly elucidated.

There are several possible ways in which E-5166 inhibits PI turnover stimulated by EGF: (1) E-5166 may modulate the EGF receptors, (2) E-5166 may inhibit phospholipase C activity, or (3) E-5166 may inhibit PI resynthesis.

In our prelabeling system, it was observed that E-5166 rather inhibited [^{3}H]glycerol labeling of DG, while it stimulated [^{3}H]inositol labeling of PI. In addition, it also stimulated [^{3}H]glycerol labeling of PA and PI plus PS, and [^{32}P] labeling of PA and PI plus PS (data not shown). Since it is reported that retinoids specifically enhance the number of EGF receptors [19], it is not likely that E-5166 may modulate them. E-5166 may have profound effects on phospholipid metabolism of pig epidermis.

Our present report suggests that E-5166 inhibits the EGF-stimulated PI breakdown, the EGF-stimulated AA release, and probably the synthesis of AA metabolites. This inhibitory effect of E-5166 may account for the clinical effectiveness on inflammatory skin diseases.

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